INHIBITION OF FARNESYLTRANSFERASE ACTIVITY IN PLANTS

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims priority to USSN 60/102,569, which is incorporated herein by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with government support under grant MCB-9506191 awarded by the National Science Foundation and grant 94-ER20148 awarded by the Department of Energy. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention is directed to improving the ability to grow plant under conditions of limited availability of water. In particular, it relates to methods of inhibiting the action of the enzyme farnesyltransferase ("FTase" or "Ftase") in plants, and to novel compositions, including plants in which activity of this enzyme is reduced.

BACKGROUND OF THE INVENTION

Agriculture depends in large part on providing plants with appropriate amounts of water. Within this context, lack of water is more often a problem than is too much. Large areas of the U.S. receive too little rainfall to support many of the important cash crops, and even areas with normally abundant rainfall experience periods of drought.

These problems have long been combated by providing water to augment what nature supplies. Thus, irrigation troughs and hoses and overhead spray arms are typically used where rainfall is not sufficient or sufficiently frequent for the crops grown. Additionally, the availability of water frequently limits the plants which can be grown in a particular locale.

Farmers have also approached resolving this problem by growing plants which need less water to thrive. Often, this has meant changing the type of crop grown to reflect the amount of water available. Effort has also been devoted to developing plants which require less water to grow. Developing such plants has traditionally been

cumbersome and required trial and error and the repetitious selection and crossing of plants with desired characteristics, followed by time consuming testing of the subsequent offspring.

SUMMARY OF THE INVENTION

The invention relates to plants which have reduced farnesyltransferase activity or reduced expression of farnesyltransferase. The invention further provides methods of reducing farnesyltransferase activity in plants.

The invention provides a plant comprising recombinant expression cassette, wherein the expression cassette comprises a promoter and a nucleic acid sequence encoding an inhibitor of farnesyltransferase. In some embodiments, the promoter is a promoter specifically activated in guard cells. The inhibitor can be a protein, or can be a nucleic acid, such as a ribozyme. Cells transformed with such a recombinant expression cassette can be regenerated into plants with reduced (inhibited) farnesyltransferase activity. Plants containing such nucleic acid constructs can be used to produce seeds which will grow into plants themselves having reduced farnesyltransferase activity.

The invention further relates to methods of inhibiting farnesyltransferase in a plant by introducing into the plant a recombinant expression cassette, wherein the expression cassette comprises a promoter operably linked to a nucleic acid sequence encoding an inhibitor of farnesyltransferase, whereby the inhibitor is expressed in the plant. In some embodiments, the promoter is a promoter specifically activated in guard cells. The inhibitor can be a protein, or can be a nucleic acid, such as a ribozyme. The introduction into the plant can be, for example, by *Agrobacterium*, by a "biolistic" technique, such as shooting DNA-coated microspheres or other microparticles into the plant, or by sexual cross. Cells such as protoplasts can be microinjected and then used to regenerate a plant with reduced farnesyltransferase activity. Farnesyltransferase activity can also be inhibited by introducing into a plant an isolated nucleic acid complementary to at least 30 nucleotides of a nucleic acid sequence encoding farnesyltransferase, such as SEQ ID NO:1 of WO 99/06580. The isolated nucleic acid can, for example, further be complementary to 100 or more nucleotides of a nucleic acid sequence encoding a farnesyltransferase, to 250 or nucleotides, or to 1000 or more nucleotides.

The invention further provides methods of inhibiting farnesyltransferase in a plant, which method comprises contacting the plant with an inhibitor of farnesyltransferase. For example, the inhibitor can be added to water which is used to

water the plant. In one embodiment, the water can be irrigation water used to water crops in the field. The inhibitor is then taken up by the plants through the roots. In another embodiment, the inhibitor is applied to one or more parts of the plant above the soil, a process known as foliar application. In this embodiment, the inhibitor can be in solution or in a dry form, such as a powder. The inhibitor can also be in a wet or dry formulation which is added to or placed on top of the soil around the roots of a plant, similar to the process by which fertilizer is typically spread over a lawn. Examples of farnesyltransferase inhibitors which can be used are manumycin or α-hydroxyfarnesylphosphonic acid.

The invention further provides a method of producing a plant with reduced farnesyltransferase activity comprising mutating a promoter region of a nucleic acid sequence encoding a farnesyltransferase and selecting mutants with reduced expression of farnesyltransferase.

Finally, the invention provides compositions comprising an inhibitor of farnesyltransferase activity and at least one other compound or material useful in plant nurturing or cultivation. Exemplary compounds useful in these regards include pesticides, fertilizers, and mulches.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Protein farnesyltransferase inhibitors affect ABA signaling in *Arabidopsis* guard cells.

(A) The FTase inhibitor HFPA causes increased activation of ABA-regulated anion currents in *Arabidopsis* guard cells. Guard cells were treated with 10 μM ABA in the absence (- HFPA) or presence (+ HFPA) of 2 μM HFPA. Whole-cell patch clamp currents were recorded from a holding potential of +30 mV with membrane voltage steps ranging from -145 mV to +35 mV in +30 mV increments (Pei *et al.*, *Plant Cell* 9, 409 (1997)).

(B) Average magnitudes of steady-state anion currents recorded at -145 mV in the absence and presence of 2 μM HFPA with or without 10 μM ABA. Experiments were performed as in (A). Currents at the end of -145 mV voltage pulses were averaged (n = 10 to 12 guard cells for each condition).

(C) HFPA causes increased ABA sensitivity of stomatal closing. Intact leaves were floated in solution with or without 2 μM HFPA under light for 2 hr to induce stomatal opening. Then ABA at indicated concentrations was added to the bath solution to assay stomatal closing. Stomatal aperture measurements were conducted as described previously (Pei *et al.*, *Plant Cell* 9, 409 (1997)). Data from four separate experiments (n = 80 stomata per data point) are shown.

FIGURE 2. The FTase deletion mutant *era1-2* causes ABA hypersensitivity of anion channel activation and of stomatal closing.

(A) Comparison of ABA-induced stomatal closing in wild-type (WT) and the *era1-2* mutant. Data from 3 representative (n = 60 stomata per data point) of 9 experiments are shown.

(B, C) Whole-cell currents recorded in the absence (- ABA) and presence of 10 μM ABA (+ ABA) in wild-type (B) and in *era1-2* mutant (C) guard cells. 10 μM ABA was added to pipette and bath solutions (Pei *et al.*, *Plant Cell* 9, 409 (1997)). Voltage protocols in (B) and (C) were the same as in Fig. 1A.

(D) Steady-state current-voltage relationships show increased ABA activation of anion currents in *era1-2* guard cells compared to those in wild-type guard cells. Recordings were performed as in (B) and (C) (n = 14 to 27 cells averaged per curve). Symbols in (D) are as illustrated in (B) and (C).

FIGURE 3. ERA1 FTase deletion suppresses the ABA-insensitive (semi-) dominant mutations in *era1/abi1* and era1/*abi2* homozygous double mutants.

(A) ABA activation of anion channel currents was analyzed in the absence (- ABA) and presence of 10 μM ABA (+ ABA) in *era1/abi1* double mutant guard cells and compared to *abi1* mutant guard cells.

(B) ABA activation of anion channel currents was analyzed in the absence and presence of 10 μM ABA in era1/*abi2* double mutant guard cells and compared to *abi2* mutant guard cells. Time and current scale bars refer to both (A) and (B).

(C, D) Steady-state current-voltage relationships as recorded in (A) and (B) respectively. Symbols in (C) and (D) correspond to those in (A) and (B), respectively (n = 5 cells for *abi1* and *abi2*; n = 10 to 16 cells for *era1/abi1* and era1/*abi2* per condition).

(E) ERA1 FTase mutation partially suppresses the ABA insensitivity of stomatal closing in the *abi2* mutant. ABA-induced stomatal closures in *abi2* and era1/*abi2* double mutant were compared. Stomatal apertures of *abi2*, Colombia WT and era1/*abi2* were normalized with respect to the apertures in the absence of ABA respectively for comparison. The abbreviation "r.u." stands for "relative unit." Data from three separate experiments are illustrated (n = 60 stomata per bar).

DETAILED DESCRIPTION

I. Introduction

The present invention is directed to plant cultivation methods. In particular, it relates to new methods for controlling stomatal closing mechanisms to decrease desiccation in plants during drought. The methods are useful, for example, in agricultural production and horticultural preservation. The methods can be carried out by, for example, producing transgenic plants comprising nucleic acids encoding an inhibitor of farnesyltransferase. Alternatively, a composition comprising a substance that induces a hypersensitive response to abscisic acid (*e.g.*, an inhibitor of farnesyltransferase) can be applied to a plant. Additionally, seeds comprising nucleic acids encoding inhibitors of farnesyltransferase can be produced by plants transformed with such nucleic acids. Such seeds are especially suitable for use in areas with limited availability of water.

Protein farnesylation, a post-translational modification process, mediates the carboxyl-terminal lipidation of specific cellular signaling proteins including Ras, GTPases, trimeric GTP-binding protein, nuclear lamin B and yeast mating pheromone a-factor (Clarke *et al.*, *Annu. Rev. Biochem.* 61, 355 (1992); F. L. Zhang and P. J. Casey, *ibid.* 65, 241 (1996); W. R. Schafer and J. Rine, *Annu. Rev. Genet.* 26, 209 (1992); J. A. Glomset and C. C. Farnsworth, *Annu. Rev. Cell Biol.* 10, 181 (1994)). In each of these cases, farnesylation increases membrane association and cellular activity of these proteins. Thus, farnesylation plays an essential role in signal transduction cascades of yeast and mammalian cells (Clarke *et al.*, *Annu. Rev. Biochem.* 61, 355 (1992); F. L. Zhang and P. J. Casey, *ibid.* 65, 241 (1996); W. R. Schafer and J. Rine, *Annu. Rev. Genet.* 26, 209 (1992); J. A. Glomset and C. C. Farnsworth, *Annu. Rev. Cell Biol.* 10, 181 (1994)).

In plant cells, farnesyltransferase ("FTase") activities have been identified, and changes in FTase activity during cell growth and division have been demonstrated (Randall *et al.*, *Plant Cell* 5, 433 (1993); Parmryd *et al.*, *Eur. J. Biochem.* 234, 723 (1995); Morehead *et al.*, *Plant Physiol.* 109, 277 (1995); Schmitt *et al.*, *ibid.* 112, 767 (1996); Yang *et al.*, *ibid.* 101, 667 (1993); Qian *et al.*, *Plant Cell* 8, 2381 (1996); Yalovsky *et al.*, *Mol. Cell. Biol.* 17, 1986 (1997)). In *Arabidopsis*, recessive mutations in the ERA1 gene, which encodes the FTase β-subunit, were identified and have been shown to prolong seed dormancy due to an enhanced response to ABA (Cutler *et al.*, *Science* 273, 1239 (1996)). This suggests farnesylation may be essential for negative regulation of ABA signaling in seeds.

Desiccation of plants during drought can be detrimental to agricultural production. The phytohormone abscisic acid (ABA) reduces water loss by triggering stomatal pore closure in leaves, a process requiring ion channel modulation by cytoplasmic proteins. Reduction of water loss can reduce plant desiccation and increase their resistance to, and ability to thrive in, drought. Plants lose over 90 % of water via transpiration through stomatal pores formed by pairs of guard cells on the leaf surface.

The hormone ABA is synthesized in response to drought stress and triggers a signaling cascade in guard cells which results in stomatal closing (A. M. Hetherington and R. S. Quatrano, *New Phytol.* 119, 9 (1991); J. A. D. Zeevaart and R. A. Creelman, *Annu. Rev. Pl. Physiol. Pl. Mol. Biol.* 39, 439 (1988); J. Leung and J. Giraudat, *ibid.* 49, 199 (1998). Studies have indicated that activation of anion channels in the plasma membrane of guard cells is required during ABA-induced stomatal closing (S. M. Assmann, *Annu. Rev. Cell Biol.* 9, 345 (1993); J. M. Ward, *et al.*, *Plant Cell* 7, 833 (1995); E. A. C. MacRobbie, *J. Exp. Bot.* 48, 515 (1997); J. I. Schroeder and S. Hagiwara, *Nature* 338, 427 (1989); Hedrich *et al.*, *EMBO J.* 9, 3889 (1990); Schmidt *et al.*, *Proc. Natl. Acad. Sci.* USA 92, 9535 (1995); A. Schwartz *et al.*, *Plant Physiol.* 109, 651 (1995); Grabov *et al.*, *Plant J.* 12, 203 (1997); Pei *et al.*, *Plant Cell* 9, 409 (1997)). Coupling of intracellular signaling proteins to membrane ion channels is essential for this ABA-mediated response (S. M. Assmann, *supra*; J.M. Ward, *et al., supra*; E. A. C. MacRobbie, *J. Exp. Bot.* 48, 515 (1997); Pei *et al.*, *supra*).

We have now discovered that cytoplasmic regulators are linked to ABA regulation of ion channels via farnesylation. More specifically, we have discovered that this is a mechanism for regulating guard cells in closing stomata. Inhibiting farnesylation

therefore results in a reduction of water loss by transpiration and enhances the ability of a plant to withstand desiccation or drought.

After defining some of the terms used herein, methods of inhibiting farnesyltransferase functioning or expression are discussed, along with the creation of transgenic plants and cells and tissue cultures expressing a farnesyltransferase inhibitor. Antisense constructs which inhibit farnesyltransferase are also discussed. Finally, examples are set forth.

II. **Definitions**

The phrase "nucleic acid sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role.

The term "promoter" refers to regions or sequences located upstream, downstream, or both, from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. A "guard cell promoter" is one capable of initiating transcription in plant guard cells. Preferably, guard cell promoters initiate transcription in guard cells, but do not initiate significant transcription in other plant cell types or tissues.

The term "plant" includes whole plants, shoot vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit (the mature ovary), plant tissue (*e.g.* vascular tissue, ground tissue, and the like) and cells (*e.g.* guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants which can be used in the methods of the invention involving transformation is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae, and includes trees, crops grown as human and animal food, plants grown for fiber, and plants grown as ornamentals, such as flowers. It further includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous. In preferred embodiments, the plants are plants with guard cells.

"Foliar application" refers to application of a substance to portions of the plant above the surface of the soil. For example, many agricultural chemicals are applied to plants in a spray which wets the leaves, stems, fruit, and other parts of the plant above the soil. By contrast, substances in irrigation water are typically taken up by a plant through the root system.

A polynucleotide sequence is "heterologous to" an organism or a second polynucleotide sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is not naturally associated with the promoter (*e.g.* a genetically engineered coding sequence or an allele from a different ecotype or variety).

A polynucleotide "exogenous to" an individual plant is a polynucleotide which is introduced into the plant by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation, biolistic methods, electroporation, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T_1 (*e.g.* in *Arabidopsis* by vacuum infiltration) or R_0 (for plants regenerated from transformed cells *in vitro*) generation transgenic plant. Transgenic plants that arise from sexual cross or by selfing are descendants of such a plant.

A "farnesyltransferase nucleic acid" or "farnesyltransferase polynucleotide sequence" is a subsequence or full length polynucleotide sequence which encodes a plant farnesyltransferase, as described for instance, by McCourt *et al.*, in WO 99/06580, which is hereby incorporated by reference. For example, the term refers to a subsequence or full length polynucleotide, or complement thereof, of SEQ ID NO:1 of WO 99/06580. SEQ ID NO:1 sets forth the sequence of a farnesyltransferase gene known as "*ERA*-1," from *Arabidopsis thaliana*. Genes encoding farnesyltransferases in yeast and a number of plants, including peas, tomato, and tobacco, are known in the art. *See, e.g.*, Yang, Z., *et al.*, Plant Physiol. 101:667-674 (1993), National Center for Biotechnology Information (NCBI) Entrex accession no. L08664 (pea FTase beta subunit); Yalovsky, S., *et al.*, Mol. Cell Biol. 17:1986-1994 (1997), NCBI Entrez accession nos. U83708 and U83707 (tomato FTase beta and alpha subunits); Powers, S., PNAS (USA) 88:11373-11377 (1991) (yeast FTase alpha subunit); Zhou, D. *et al.*, Plant J., 12:921-930 (1997); Danjoh, I., and Fujiyama, A., Eur. J. Biochem., 236:847-851 (1996).

An "inhibitor" of farnesyltransferase refers to a compound which results in a decrease of farnesyltransferase expression or activity. Examples of such decreased activity or expression include the following: (a) farnesyltransferase activity or expression of a gene encoding farnesyltransferase is decreased below the level of that in wild-type, non-transgenic control plants (i.e. the quantity of farnesyltransferase activity or expression of a gene encoding farnesyltransferase is decreased); (b) farnesyltransferase activity or expression is not detected in an organ, tissue or cell where it is normally detected in wild-type, non-transgenic control plants (*i.e.* spatial distribution of farnesyltransferase activity or expression is decreased); (c) farnesyltransferase activity or expression in an organ, tissue or cell occurs for a shorter period than in a wild-type, non-transgenic controls (*i.e.* duration of farnesyltransferase activity or expression is decreased). Use of the word "inhibitor" alone herein refers to an inhibitor of farnesyltransferase activity or of the expression of a gene encoding farnesyltransferase, as required by context.

As used herein, the term "encoding" refers, with respect to a DNA molecule, at least to transcription into an RNA molecule or ribozyme, and can further comprise translation of the DNA into a protein.

Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. When percentage of sequence identity is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full

mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated according to, e.g., the algorithm of Meyers & Miller, *Computer Applic. Biol. Sci.* 4:11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, CA).

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to sequences or subsequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity when aligned for maximum correspondence over a comparison window as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition also refers to the complement of a test sequence, which has substantial sequence or subsequence complementarity when the test sequence has substantial identity to a reference sequence.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and

TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al, supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value;

the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, in a nucleic acid, peptide, polypeptide, or protein sequence which alters a

single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another: conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(*see, e.g.*, Creighton, *Proteins* (1984)).

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below.

The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, highly stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific

sequence at a defined ionic strength pH. Low stringency conditions are generally selected to be about 15-30 °C below the T_m. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 time background hybridization.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions.

In the present invention, genomic DNA or cDNA comprising farnesyltransferase nucleic acids of the invention can be identified in standard Southern blots under stringent conditions using the nucleic acid sequences disclosed in international publication WO 99/06580, and especially SEQ ID NO:1. For the purposes of this disclosure, suitable stringent conditions for such hybridizations are those which include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and at least one wash in 0.2X SSC at a temperature of at least about 50°C, usually about 55°C to about 60°C, for 20 minutes, or equivalent conditions. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

A further indication that two polynucleotides are substantially identical is if the reference sequence, amplified by a pair of oligonucleotide primers, can then be used as a probe under stringent hybridization conditions to isolate the test sequence from a cDNA or genomic library, or to identify the test sequence in, *e.g.*, an RNA gel or DNA gel blot hybridization analysis.

III. Inhibition of farnesyltransferase activity

A number of inhibitors of farnesyltransferase activity in plants are known and can be used in the methods of the invention. For example, two such inhibitors, α-hydoxyfarnesylphosphonic acid (HFPA) and manumycin, were used in the studies described in the Examples. In addition to these inhibitors, farnesyltransferase has been the subject of considerable research which has resulted in the development of a number of peptide and peptidomimetic compounds which inhibit the enzyme. Since 1998 alone, for example, U.S. Patents 5,925,641; 5,932,590, 5,919,780; 5,914,341; 5,872,135; 5,856,321; 5,856,310; 5,852,010; 5,834,434; and 5,705,686, have issued on novel farnesyltransferase inhibitors. The homology of the farnesyltransferase gene family, and the fact plant FTase can be substituted for endogenous FTase in FTase-deficient yeast (*see, e.g.*, Yalovsky *et al.*, Mol. Cell Biol. 17:1986-1994 (1997)), lead us to expect that farnesyltransferase inhibitors effective in one organism will have an inhibitory effect on FTases of other organisms. Any particular inhibitor can be tested by routine assays in the art, and the drought assays discussed below, to determine if it is effective in inhibiting farnesylation in a plant of interest.

Inhibitors of farnesyltransferase activity can be used to inhibit such activity in plants in a variety of ways. First, nucleic acids encoding peptide inhibitors can be introduced into plants by methods discussed in the preceding section. In preferred embodiments, such nucleic acids are introduced in the form of recombinant expression cassettes, containing an appropriate promoter. In more preferred embodiments, the promoter used is one specific for expression in leaves. In particularly preferred embodiments, the promoter used is one which specifically or preferentially promotes expression in guard cells. One such particularly preferred promoter is KAT1, which has been shown in transgenic plants to drive expression primarily in guard cells (*see*, Nakamura, R., *et al.*, Plant Physiol. 109:371-374 (1995). Another particularly preferred promoter is the truncated 0.3 kb 5' proximal fragment of potato ADP-glucose pyrophosphorylase, which has been shown to drive expression exclusively in guard cells of transgenic plants. *See*, *e.g.*, Muller-Rober, B., *et al.*, Plant Cell, 6:601-612 (1994).

Persons of skill in the art are aware of a variety of methods for introducing heterologous nucleic acids into plants. Such methods include *Agrobacterium*-mediated transformation, biolistic methods, electroporation, and the like. In one class of embodiments, plant cells are transformed with the nucleic acids and regenerated into

plants from the transformed cells. Further, if the technique for transforming the cell results in integration of the heterologous nucleic acids into the genome of the cell, seeds resulting from the regenerated plants will contain the introduced nucleic acids. Traditional horticultural techniques, such as sexual crosses, can also be used to introduce desired genes into plants.

Second, peptide, peptidomimetic, or other inhibitors can be applied to the plants. Conveniently, this can be done by adding the inhibitors to irrigation water or to water or to foliar sprays. Inhibitors can be applied alone or in mixture with other plant hormones, fertilizers, pesticides or fungicides. The inhibitor can be applied in a mixture with a carrier or, if necessary, other auxiliary agents to form any one of the standard types of preparations commonly used in agriculture, for example, a dry blend, granules, a wettable powder, an emulsion, an aqueous solution and the like.

Suitable solid carriers are clay, talc, kaolin, bentonite, terra abla, calcium carbonate, diatomaceous earth, silica, synthetic calcium silicate, kieselguhr, dolomite, powdered magnesia, Fuller's earth, gypsum and the like. Solid compositions can also be in the form of dispersible powders or grains, comprising, in addition to the inhibitor, a surfactant to facilitate the dispersion of the powder or grains in liquid. Liquid compositions include solutions, dispersions or emulsions containing the auxins together with one or more surface-active agents such as wetting agents, dispersing agents, emulsifying agents, or suspending agents. In those applications in which the compounds are applied as a foliar spray, surface active agents are preferably used.

Generally, any number of surfactants may be used consistent with the purpose of this constituent. For example the surfactant can comprise a nonionic, anionic, cationic, or zwitterionic surfactant. The surfactant can be formulated with the inhibitor as formulated or, alternatively, the surfactants can be introduced during application to the plant. In such an instance, regardless of whether the application is conducted via automated or manual means, the surfactant can be combined with the inhibitor prior to application or codispensed separately during application. The average molecular weight of useful surfactants ranges from about 100 to about 4000.

Cationic surfactants useful in compositions of the invention include, for example, amine ethoxylates, amine oxides, mono- and dialkylamines, imidazolinium derivatives, and alkylbenzyldimethylammonium halides.

Nonionic surfactants useful in the context of this invention are generally polyether (also known as polyalkylene oxide, polyoxyalkylene or polyalkylene glycol) compounds. More particularly, the polyether compounds are generally polyoxypropylene or polyoxyethylene glycol compounds.

Anionic surfactants useful with the invention comprise, for example, alkyl carboxylates, linear alkylbenzene sulfonates, paraffin sulfonates and secondary n-alkane sulfonates, sulfosuccinate esters and sulfated linear alcohols.

Zwitterionic or amphoteric surfactants useful with the invention comprise α-N-alkylaminopropionic acids, n-alkyl-α-iminodipropionic acids, imidazoline carboxylates, amine oxides, sulfobetaines and sultaines.

Although the surfactant can be present in the composition in any useful amount, in preferred embodiments, it is present in an amount from about 0.1% to about 25%, more preferably from about 0.1% to about 10% and more preferably still from about 0.5% to about 5%. A surfactant is present in the compositions of the invention in a useful amount when it facilitates the dissolution of the inhibitor, or enhances its uptake by the plant, or both, or its effectiveness in inducing the desired response, or provides all three of these effects.

Compositions used in the methods of the invention can also contain suspending agents. Suitable suspending agents are, for example, hydrophilic colloids, for example polyvinylpyrollidone and sodium carboxymethylcellulose, and vegetable gums, such as gum acacia and gum tragacanth.

Aqueous solutions, dispersions or emulsions may be prepared by dissolving the inhibitor in water or an organic solvent which can, if desired, contain one or more surface active, sticking, wetting, dispersing, or emulsifying agents. Suitable organic solvents are, for example, alcohols, hydrocarbons, oils and sulfoxides. In embodiments using alcohols, methanol, isopropyl alcohol, propylene glycol and diacetone alcohol are preferred. In embodiments using oils, petroleum oils are preferred. Of the sulfoxides, dimethylsulfoxide is preferred.

The inhibitor can also be microencapsulated. Microcapsules containing the desired inhibitor can be prepared by co-acervation; or, more preferably, by stirred interfacial polymerization of (for example) an isocyanate/diamine system. The resulting microcapsules may be used as an aqueous suspension.

Inhibitors which are to be used in the form of aqueous solutions, dispersions or emulsions are generally supplied in the form of a concentrate containing a high proportion of the inhibitor, and the concentrate is then diluted with water before use. These concentrates are usually required to withstand storage for prolonged periods and after such storage, to be capable of dilution with water in order to form aqueous preparations which remain homogeneous for a sufficient time to enable them to be applied by conventional spray equipment. In general, concentrates can conveniently contain from 10-60 percent by weight of the inhibitor. Dilute preparations ready for use may contain varying amounts of the inhibitor. The practitioner can observe plants treated with the inhibitor and decrease the dilution of the inhibitor if it appears the plants are showing signs of wilting or browning.

In carrying out the methods of the invention, an "effective amount" of a farnesyltransferase inhibitor is applied to the plants. One of skill will recognize that an effective amount of an inhibitor will vary and will depend upon a number of factors including, for example, the particular inhibitor and formulation selected for use, the timing of the application, whether the compound is to be applied for foliar or root uptake, and the plant species whose growth is to be regulated.

IV. Inhibition of Expression of the Farnesyltransferase Gene

A. Antisense Technology

A number of methods can be used to inhibit the expression of genes encoding farnesyltransferase in plants. For instance, antisense technology can be conveniently used. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the antisense strand of RNA will be transcribed. Preferably, the promoter is one which drives expression preferentially in leaf cells. Most preferably, the promoter preferentially drives expression in guard cells. The construct is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has been suggested that antisense suppression can act at all levels of gene regulation including suppression of RNA translation (*see*, Bourque *Plant Sci. (Limerick)* 105: 125-149 (1995); Pantopoulos, *in: Progress in Nucleic Acid Research and Molecular Biology*, Vol. 48. Cohn, W. E. and K. Moldave (Ed.). Academic Press, Inc., San Diego,

California, p. 181-238; Heiser *et al. Plant Sci. (Shannon)* 127: 61-69 (1997)) and by preventing the accumulation of mRNA which encodes the protein of interest, *(see,* Baulcombe *Plant Mol. Bio.* 32:79-88 (1996); Prins and Goldbach *Arch. Virol.* 141: 2259-2276 (1996); Metzlaff *et al. Cell* 88: 845-854 (1997), Sheehy et al., *Proc. Nat. Acad. Sci. USA*, 85:8805-8809 (1988), and Hiatt et al., U.S. Patent No. 4,801,340). For example, constructs to the *ERA-1* gene can be developed using the sequence of SEQ ID NO:1 set forth in WO 99/06580.

The nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the endogenous farnesyltransferase gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other genes within a family of genes exhibiting identity or substantial identity to the target gene.

For antisense suppression, the introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA. Generally, higher identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments may be equally effective. Normally, a sequence of between about 30 or 40 nucleotides and about the full length sequence should be used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of about 500 to about 3500 nucleotides is especially preferred.

A number of gene regions can be targeted to suppress farnesyltransferase gene expression. The targets can include, for instance, the coding regions, introns, sequences from exon/intron junctions, 5' or 3' untranslated regions, and the like.

Inhibiting the expression of either the α-subunit or the β-subunit of farnesyltransferase will result in inhibition of the enzyme's activity. Typically, the β-subunit is the one selected since it is more specific to the function of this enzyme. In contrast, the α-subunit of farnesyltransferase is involved in other cellular prenylation reactions. Thus, if expression of this subunit is to be inhibited, it is desirable to do so in a way which targets the inhibition to guard cells or to other selected cell types in which the inhibition of farnesylation is desired. This can be done, for example, by using a

promoter which is preferentially or primarily expressed in the cell type of interest. The sequences for subunits for plants such as pea, tomato, and Arabidopsis are known in the art (see the references cited in the Definitions, supra). Suppression of the subunit of choice can readily be accomplished by such means as using antisense molecules specific for the particular subunit.

B. Co-suppression and double stranded RNA gene silencing

Several techniques have recently been developed for blocking or suppressing the expression of endogenous genes by introduction of exogenous nucleic acids. One well known method of suppression is sense co-suppression. Introduction of nucleic acid configured in the sense orientation has been recently shown to be an effective means by which to block the transcription of target genes. For examples of the use of this method to modulate expression of endogenous genes, *see,* Assaad *et al. Plant Mol. Bio.* 22: 1067-1085 (1993); Flavell *Proc. Natl. Acad. Sci. USA* 91: 3490-3496 (1994); Stam *et al. Annals Bot.* 79: 3-12 (1997); Napoli et al., *The Plant Cell* 2:279-289 (1990); and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184. Post-transcriptional gene silencing induced by introduction of transgenes blocks expression of endogenous genes homologous to the transgene and is thought to be involved in virus resistance and genome maintenance. *See, e.g.*, Cogoni and Macino, Nature 399:166-169.

The suppressive effect may occur where the introduced sequence contains no coding sequence *per se*, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence generally will be substantially identical to the endogenous sequence intended to be repressed. This minimal identity will typically be greater than about 65%, but a higher identity might exert a more effective repression of expression of preferred, though about 95% to absolute identity would be most preferred. As with antisense regulation, the effect should apply to any other proteins within a similar family of genes exhibiting identity or substantial identity.

For co-suppression, the introduced sequence, needing less than absolute identity, also need not be full length, relative to either the primary transcription product or fully processed mRNA. This may be preferred to avoid concurrent production of some plants which are overexpressers. A higher identity in a shorter than full length sequence compensates for a longer, less identical sequence. Furthermore, the introduced

sequence need not have the same intron or exon pattern, and identity of non-coding segments will be equally effective. Normally, a sequence of the size ranges noted above for antisense regulation is used. In addition, the same gene regions noted for antisense regulation can be targeted using co-suppression technologies.

In a further approach to blocking expression of endogenous genes, it has been found that RNA capable of forming duplexes of sense and antisense strands suppresses expression of endogenous genes more powerfully than does introduction of either strand individually in organisms as diverse as plants and worms. *See, e.g.*, Waterhouse, *et al.*, PNAS (USA) 95:13959-13964 (1998); Fire, *et al.*, Nature 391:806-811 (1998). Post-transcriptional gene silencing by introduction of double stranded RNA therefore provides another powerful tool for inhibiting farnesyltransferase activity

C. Triplex DNA formation

Oligonucleotide-based triple-helix formation can also be used to disrupt farnesyltransferase gene expression. Triplex DNA can inhibit DNA transcription and replication, generate site-specific mutations, cleave DNA, and induce homologous recombination (*see*, *e.g.*, Havre and Glazer *J. Virology* 67:7324-7331 (1993); Scanlon *et al. FASEB J.* 9:1288-1296 (1995); Giovannangeli *et al. Biochemistry* 35:10539-10548 (1996); Chan and Glazer *J. Mol. Medicine (Berlin)* 75: 267-282 (1997)). Triple helix DNAs can be used to target the same sequences identified for antisense regulation.

D. Ribozymes

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of farnesyltransferase genes. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. Thus, ribozymes can be used to target the same sequences identified for antisense regulation. In preferred embodiments, the ribozymes are targeted to specific cell types, such as guard cells or leaf cells. Conveniently, this can be achieved by introducing a DNA construct with a promoter specific for the cell type of interest.

A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs which are capable of self-cleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch or viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus. The design and use of target RNA-specific ribozymes is described in Zhao and Pick, *Nature* 365:448-451 (1993); Eastham and Ahlering, *J. Urology* 156:1186-1188 (1996); Sokol and Murray, *Transgenic Res.* 5:363-371 (1996); Sun *et al., Mol. Biotechnology* 7:241-251 (1997); and Haseloff *et al., Nature*, 334:585-591 (1988).

E. Modification and knocking out of endogenous genes

In addition to blocking expression of endogenous genes, as discussed above, methods for introducing genetic mutations into the genes can also be used to select for plants with decreased farnesyltransferase expression. In preferred embodiments, endogenous genes encoding farnesyltransferase are modified by, for example, replacing the promoter with a promoter with lower activity, or with an inducible promoter so that expression of the gene can be turned off when conditions warrant.

Methods for introducing genetic mutations into plant genes and selecting plants with desired traits are well known. For instance, seeds or other plant material can be treated with a mutagenic chemical substance, according to standard techniques. Such chemical substances include, but are not limited to, the following: diethyl sulfate, ethylene imine, ethyl methanesulfonate and N-nitroso-N-ethylurea. Alternatively, ionizing radiation from sources such as, X-rays or gamma rays can be used.

Alternatively, homologous recombination can be used to induce targeted gene modifications by specifically targeting the farnesyltransferase gene *in vivo* (*see, generally*, Grewal and Klar, *Genetics* 146: 1221-1238 (1997) and Xu *et al.*, *Genes Dev.* 10: 2411-2422 (1996)). Homologous recombination has been demonstrated in plants (Puchta *et al.*, *Experientia* 50: 277-284 (1994), Swoboda *et al.*, *EMBO J.* 13: 484-489 (1994); Offringa *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 7346-7350 (1993); and Kempin *et al. Nature* 389:802-803 (1997)).

In applying homologous recombination technology to farnesyltransferase genes, mutations in selected portions of a farnesyltransferase gene

sequence (including 5' upstream, 3' downstream, and intragenic regions) are made *in vitro* and then introduced into the desired plant using standard techniques. Since the efficiency of homologous recombination is known to be dependent on the vectors used, use of dicistronic gene targeting vectors as described by Mountford *et al., Proc. Natl. Acad. Sci. USA* 91: 4303-4307 (1994); and Vaulont *et al., Transgenic Res.* 4: 247-255 (1995) are conveniently used to increase the efficiency of selecting for altered farnesyltransferase gene expression in transgenic plants. The mutated gene will interact with the target wild-type gene in such a way that homologous recombination and targeted replacement of the wild-type gene will occur in transgenic plant cells, resulting in suppression of farnesyltransferase activity.

Alternatively, oligonucleotides composed of a contiguous stretch of RNA and DNA residues in a duplex conformation with double hairpin caps on the ends can be used. The RNA/DNA sequence is designed to align with the sequence of the target farnesyltransferase gene and to contain the desired nucleotide change. Introduction of the chimeric oligonucleotide on an extrachromosomal T-DNA plasmid results in efficient and specific farnesyltransferase gene conversion directed by chimeric molecules in a small number of transformed plant cells. This method is described in Cole-Strauss *et al. Science* 273:1386-1389 (1996) and Yoon *et al. Proc. Natl. Acad. Sci. USA* 93: 2071-2076 (1996).

Finally, endogenous genes can also be "knocked-out" by transposons or T-DNA insertion. These are random insertions into the genome that disrupt the activity of the endogenous gene into which the transposon or T-DNA is inserted. Since the insertions are random, the subject cells or plants are then screened for those in which farnesyltransferase activity has been reduced.

V. Other means for inhibiting farnesyltransferase activity

Farnesyltransferase activity may be modulated by eliminating the proteins that are required for farnesyltransferase cell-specific gene expression. Thus, expression of regulatory proteins, or the sequences that control farnesyltransferase gene expression, or both, can be modulated using the methods described here.

Another strategy is to inhibit the ability of a farnesyltransferase protein to interact with itself or with other proteins. This can be achieved, for instance, using antibodies specific to farnesyltransferase. In this method, cell-specific expression of

farnesyltransferase -specific antibodies is used to inactivate functional domains through antibody:antigen recognition (*see*, Hupp *et al.*, *Cell* 83:237-245 (1995)). Interference of activity of a farnesyltransferase interacting protein can be applied in a similar fashion. Alternatively, dominant negative mutants of farnesyltransferase can be prepared by expressing a transgene that encodes a truncated farnesyltransferase protein. Use of dominant negative mutants to inactivate target genes in transgenic plants is described in Mizukami *et al.*, *Plant Cell* 8:831-845 (1996).

VI. Preparation of recombinant vectors

To use isolated nucleic acid sequences for use in the techniques requiring them, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. *See, e.g.*, Weising *et al.*, *Ann. Rev. Genet.* 22:421-477 (1988). A DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

For example, for overexpression of an inhibitor, a plant promoter fragment may be employed which will direct expression of the gene in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumafaciens*, and other transcription initiation regions from various plant genes known to those of skill. Such genes include for example, *ACT11* from *Arabidopsis* (Huang *et al. Plant Mol. Biol.* 33:125-139 (1996)), *Cat3* from *Arabidopsis* (GenBank No. U43147, Zhong *et al.*, *Mol. Gen. Genet.* 251:196-203 (1996)), the gene encoding stearoyl-acyl carrier protein desaturase from *Brassica napus* (GenBank No. X74782, Solocombe *et al. Plant Physiol.* 104:1167-1176 (1994)), *GPc1* from maize (GenBank No. X15596, Martinez *et al. J. Mol. Biol* 208:551-565 (1989)), and *Gpc2* from maize (GenBank No. U45855, Manjunath *et al.*, *Plant Mol. Biol.* 33:97-112 (1997)).

Alternatively, the plant promoter may direct expression of an inhibitor of farnesyltransferase in a specific tissue, organ or cell type (*i.e.* tissue-specific promoters) or may be otherwise under more precise environmental or developmental control (*i.e.* inducible promoters). Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, elevated temperature, the presence of light, or spraying with chemicals, plant hormones, or both. One of skill will recognize that a tissue-specific promoter may drive expression of operably linked sequences in tissues other than the target tissue. Thus, as used herein a tissue-specific promoter is one that drives expression preferentially in the target tissue or cell type, but may also lead to some expression in other tissues as well. Most preferably, the tissue-specific promoter drives expression of the gene preferentially in plant guard cells.

If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences (*e.g.*, promoters or coding regions) from genes of the invention will typically comprise a marker gene that confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

VII. Production of transgenic plants

DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski et al. *Embo J.* 3:2717-2722 (1984). Electroporation techniques are described in Fromm et al. *Proc. Natl. Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein *et al.*

Nature 327:70-73 (1987). Typically, in such techniques, DNA is coated onto a suitable microparticle and "shot" into tissue of the target organism by a focused blast of a gas or a liquid carrier.

Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example, Horsch *et al.*, *Science* 233:496-498 (1984); Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* 80:4803 (1983) and *Gene Transfer to Plants*, Potrykus, *ed.* (Springer-Verlag, Berlin 1995). In preferred embodiments, the *A. tumefaciens*-mediated transformation occurs by vacuum infiltration or by the so-called "plant dunking" technique, in which the plant of interest is immersed in an *A. tumefaciens*-containing solution.

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype such as decreased farnesyltransferase activity. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans *et al.*, *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, pp. 124-176, MacMillilan Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee *et al.*, *Ann. Rev. of Plant Phys.* 38:467-486 (1987).

The nucleic acids of the invention can be used to confer desired traits on essentially any plant. Thus, the invention has use over a broad range of plants, including species from the genera *Anacardium, Arachis, Asparagus, Atropa, Avena, Brassica, Chlamydomonas, Chlorella, Citrus, Citrullus, Capsicum, Carthamus, Cocos, Coffea, Cucumis, Cucurbita, Cyrtomium, Daucus, Elaeis, Fragaria, Glycine, Gossypium, Helianthus, Heterocallis, Hordeum, Hyoscyamus, Lactuca, Laminaria, Linum, Lolium, Lupinus, Lycopersicon, Macrocystis, Malus, Manihot, Majorana, Medicago, Nereocystis,*

Nicotiana, Olea, Oryza, Osmunda, Panieum, Pannesetum, Persea, Phaseolus, Pistachia, Pisum, Pyrus, Polypodium, Prunus, Pteridium, Raphanus, Ricinus, Secale, Senecio, Sinapis, Solanum, Sorghum, Theobromus, Trigonella, Triticum, Vicia, Vitis, Vigna, and *Zea.* In particular, the invention is useful with any plant with guard cells.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Using known procedures one of skill can screen for plants of the invention by detecting the amount of farnesyltransferase mRNA or protein in transgenic plants. Means for detecting and quantitating mRNAs or proteins are well known in the art. The plants of the invention can also be identified by detecting the desired phenotype. For instance, plants with decreased desiccation under drought conditions can be detected according to well-known techniques, including those set forth in the Examples, below.

EXAMPLES

Example 1. Farnesyltransferase is expressed in guard cells

We analyzed the effects of two competitive FTase inhibitors α-hydoxyfarnesylphosphonic acid (HFPA) (Pompliano *et al.*, *Biochemistry* 31, 3800 (1992); Gibbs *et al.*, *J. Biol. Chem.* 268, 7617 (1993)) and manumycin (Gibbs and Oliff, *Annu. Rev. Pharmacol. Toxicol.*, 37:143 (1997)); on ion channels and stomatal movements. Whole-cell patch clamp current recordings showed that in the presence of ABA, exposure of *Arabidopsis* guard cells to HEPA significantly increased ABA activation of anion currents (Fig. 1A and 1B; $P < 0.004$). In the absence of ABA, HFPA did not enhance anion currents (Fig. 1B; $P > 0.5$). Stomatal aperture measurements showed that HFPA also increased the ABA sensitivity of stomatal closing (Fig. 1C; $P < 0.001$ at 5 μM ABA). ABA-hypersensitive stomatal closure was also found by using a different FTase inhibitor, manumycin ($n = 3$ experiments, 480 stomata).

Previous studies have shown that a pea FTase β-subunit is expressed in meristematic tissues (Zhou *et al.*, *Plant J.* 12, 921 (1997)), and mRNA of the *Arabidopsis ERA1* FTase accumulates in flower buds (Cutler *et al.*, *Science* 273, 1239 (1996)). To determine whether ERA1 is also expressed in guard cells, transgenic plants expressing

ERA1 promoter-GUS constructs were analyzed in mature leaves. In intact leaves, *Arabidopsis* guard cells showed GUS activities, indicating that the *ERA1* gene is expressed in guard cells in addition to expression in other vegetative tissues.

Example 2. Farnesyltransferase affects regulation of stomatal closing

A fast-neutron mutant allele, *era1-2*, in which the entire *ERA1* gene is deleted, was used to determine whether ERA1 directly affects guard cell ABA signaling. Stomatal aperture measurements showed that the *era1-2* mutation caused ABA-hypersensitivity of stomatal closing (Fig. 2A; $P < 0.001$ at 10 μM ABA). In the absence of exogenous ABA, stomatal apertures were slightly smaller in *era1-2* than those in wild-type control plants under the imposed conditions (Fig. 2A). When the KCl concentration in solutions and light intensity were increased during stomatal opening, stomata in *era1-2* opened as wide as those in wild-type plants, but continued to show ABA hypersensitivity of stomatal closing, indicating that smaller stomatal apertures in *era1-2* might be due to sensitivity to endogenous ABA.

We examined whether the *era1* mutation affects ABA regulation of guard cell anion channels. In the absence of ABA, *era1-2* did not cause constitutive enhancement of anion currents under the imposed conditions (Figs. 2B and 2C). In the presence of 10 μM ABA, *era1-2* mutation consistently caused increased activation of anion currents compared to wild-type (Figs. 2B; $n = 30$ and 2C; $n = 49$) (in addition, ABA activation of anion channel currents was also analyzed at 1 and 50 μM ABA ($n = 23$ and 28 cells for wild-type and *era1-2*, respectively). Activation of anion currents was also potentiated in *era1-2* vs. wild-type at 1 μM ABA, whereas at 50 μM ABA both wild-type and *era1-2* responses were similar.). Current-voltage analyses showed that ABA-activated steady-state anion currents were substantially larger in *era1-2* than in wild-type guard cells (Fig. 2D; $P < 0.003$ at ±145 mV). Interestingly, transient depolarization-activated outward-rectifying K+ currents in the plasma membrane of guard cells were enhanced by the *era1* mutation in the absence of ABA. For example, peak currents at +100 mV were 254 (24 pA ($n = 18$) in wild-type and 411 (36 pA ($n = 15$) in *era1-2* guard cells.

The above data show that deletion of the *ERA1* farnesyltransferase gene causes ABA hypersensitivity of anion channel activation and stomatal closing. The findings that FTase inhibitors mimic the *ERA1* deletion mutation in wild-type plants (Fig.

1) suggest that ABA hypersensitivity in *era1-2* is not due to a long term effect of FTase deletion during guard cell maturation. Rather these data suggest that farnesyltransferases modulate a negative regulation pathway of guard cell ABA signaling.

The ABA insensitive mutant loci *abi1* and *abi2* (Koornneef *et al.*, *Physiol. Plant.* 61, 377 (1984); R. R. Finkelstein and C. R. Somerville, *Plant Physiol.* 94, 1172 (1990)) encode type 2C protein phosphatases (PP2C) (J. Leung *et al.*, *Science* 264, 1448 (1994); Meyer *et al.*, ibid. p. 1452; Leung *et al.*, *Plant Cell* 9, 759 (1997); Rodriguez *et al.*, *FEBS Let.* 421, 185 (1998)). Recent studies have led to models in which these PP2Cs may function as negative regulators in ABA signaling (Pei *et al.*, *supra*; Armstrong *et al.*, *Proc. Natl. Acad. Sci. USA*, 92, 9520 (1995); *J. Sheen, ibid.* 95, 975 (1998)). *Abi1* and *abi2* do not have farnesylation consensus sequences. To test whether the *era1* and *abi* mutations interact genetically, homozygous double mutants of *era1/abi1* and *era1/abi2* were generated. As previously reported, activation of anion currents by ABA is impaired in the *abi1*and *abi2* mutants (Figs. 3A and 3B), consistent with impairment in ABA-induced stomatal closing (Pei *et al.*, *supra*; Koornneef *et al.*, *supra;* Finkelstein and Somerville, *supra*; Roelfsema and Prins, *Physiol. Plant.* 95, 373 (1995)). Interestingly, 10 μM ABA was sufficient to activate anion channel currents in both the *era1/abi1*(Fig. 3A) and *era1/abi2* double mutants (Fig. 3B). Steady-state current-voltage relationships clearly show that ABA activation of anion currents was restored in these two double mutants (Figs. 3C and 3D). Furthermore, ABA-induced stomatal closing was restored in the *era1/abi1* double mutant and in the *era1/abi2* double mutant (Fig. 3E). Stomatal responses of the era1/*abi2* double mutants were similar to wild-type plants, but did not fully show the era1 phenotype. Stomata of *era1/abi1* showed less ABA sensitivity than *era1/abi2*, but a clear suppression of the *abi1* phenotype. The ABA insensitivities of *abi1* and *abi2* in seed germination (Koornneef *et al.*, *supra*, Finkelstein and Somerville, *supra*) were also suppressed in these double mutants with a sensitivity sequence of *era1* > *era1/abi1* ~ *era1/ abi2* > Ler WT> Col WT > *abi1* ~ *abi2*, where Col WT is Colombia wild-type; Ler WT, Lansberg *erecta* wild-type.

Example 3. Reduced farnesyltransferase activity reduces transpiration, and hence plant water loss under drought conditions

Because deletion of the *ERA1* FTase potentiates ABA-induced anion currents and stomatal closing in epidermal strips and partially suppresses the *abi1* and

abi2 mutations, we investigated whether whole plant transpiration is reduced during drought. Both wild-type plants and *era1-2* plants were grown and watered for ~ 21 days, and then subjected to drought stress by terminating irrigation. Wild-type and *era1-2* plants showing similar developmental stages and similar number of leaves were selected for drought treatments and evaporation from soil was minimized by covering the pots. After 12 days of drought treatment, wild-type plants showed severe wiltiness and chlorosis of rosette leaves. In contrast, *era1-2* plants were turgid and leaves remained green. The *era1-2* plants also showed slowed growth, which may be partially due to increased stomatal closing and reduced carbon fixation; and/or to *ERA1* expression in several plant tissues (Cutler *et al.*, *Science* 273, 1239 (1996)). When pots were not covered, the reduced wiltiness of *era1-2* plants was visible although less pronounced. Transpiration rates of wild-type leaves were 2.8 (0.3-fold larger than those of *era1-2* plants after 10 days of drought. Stomatal apertures of both wild-type and *era1-2* decreased during drought. However, stomatal apertures of *era1-2* decreased faster and were smaller than those of wild-type during drought (*e.g.* 1.08 ± 0.05 vs 1.24 ± 0.03 μm after 4-5 days of drought; $n = 75$; $P < 0.02$). These results show that *ERA1* deletion decreases the transpiration rate of leaves and consequently slows desiccation during drought.

Protein farnesylation plays important and diverse roles in cellular processes and signal transduction cascades, which control cell growth, division, morphology and visual signaling in eukaryotic cells (Clarke *et al.*, *Annu. Rev. Biochem.* 61, 355 (1992); F. L. Zhang and P. J. Casey, *ibid.* 65, 241 (1996); W. R. Schafer and J. Rine, *Annu. Rev. Genet.* 26, 209 (1992); J. A. Glomset and C. C. Farnsworth, *Annu. Rev. Cell Biol.* 10, 181 (1994); Randall *et al.*, *Plant Cell* 5, 433 (1993); Parmryd *et al.*, *Eur. J. Biochem.* 234, 723 (1995); Morehead *et al.*, *Plant Physiol.* 109, 277 (1995); Schmitt *et al.*, *ibid.* 112, 767 (1996); Yang *et al.*, *ibid.* 101, 667 (1993); Qian *et al.*, *Plant Cell* 8, 2381 (1996); Yalovsky *et al.*, *Mol. Cell. Biol.* 17, 1986 (1997)). Competitive FTase inhibitors, as used here (Fig. 1), have been shown to reduce Ras-mediated tumor growth (J. B. Gibbs and A. Oliff, *Annu. Rev. Pharmacol. Toxicol.* 37, 143 (1997); W. R. Schafer *et al.*, *Science* 245, 379 (1989); Hancock, *et al.*, *Cell* 57, 1167 (1989); Barrington *et al.*, *Mol. Cell. Biol.* 18, 85 (1998); Moasser *et al.*, *Proc. Natl. Acad. Sci. USA* 95, 1369 (1998)). However, viable null mutants in FTase genes have not yet been found in other multi-cellular eukaryotes (Clarke *et al.*, *supra*; F. L. Zhang and P. J. Casey, *ibid.*; W. R.

Schafer and J. Rine, *bid*; J. A. Glomset and C. C. Farnsworth, *supra*) and ion channel modulation by farnesyltransferases has not yet been reported. In plants, roles of protein farnesylation have been demonstrated in cell cycle regulation (Randall *et al.*, *supra*; Parmryd *et al.*, *supra*; Morehead *et al.*, *supra*; Schmitt *et al.*, *ibid.*; Yang *et al.*, *ibid.*; Qian *et al.*, *bid*; Yalovsky *et al.*, *ibid.*; Zhou *et al.*, *Plant J.* 12, 921 (1997)) and in seed germination (Cutler *et al.*, *Science* 273, 1239 (1996)). The only plant protein of known function shown to be farnesylated *in vivo* thus far is ANJ1, which is a homolog of the bacterial molecular chaperone DnaJ (Zhu *et al.*, *ibid.* 90, 8557 (1993)).

Although *era1* affects other signal transduction processes, we have demonstrated in guard cells a novel function for protein farnesylation in regulation of ion channels, stomatal movements and transpirational water loss via modulation of the ABA signaling cascade. Partial suppression of the ABA insensitive phenotypes of the *abi1* and *abi2* mutants by ERA1 deletion suggests that the target of the ERA1 FTase may function downstream or parallel to these ABI protein phosphatases. We propose that the ERA1 farnesyltransferase plays a major role in linking undetermined soluble negative regulatory proteins to plasma membrane ion channel regulation in guard cells. Modulation of ERA1 or its targets, specifically in guard cells or other cell types, will allow further analysis of ERA1 effects on gas exchange, growth and development. In conclusion, we provide biophysical, genetic, cell biological, pharmacological and whole plant physiological evidence for a molecular mechanism causing ABA hypersensitivity in guard cell signaling.

Example 4. Stomatal aperture measurements

Stomatal aperture measurements were conducted as described previously (Pei *et al.*, *Plant Cell* 9, 409 (1997)). Detached rosette leaves were floated in solutions containing 20 mM KCl, 1 mM $CaCl_2$, 5 mM Mes-KOH, pH 6.15, and exposed to light at a fluency rate of 300 μmol m^{-2} s^{-1}; subsequently, the indicated concentrations of ABA and/or 2 μM HFPA or 5 μM manumycin were added to the solutions to assay for stomatal closing; after treatments for 2 hr, stomatal apertures were observed with a digital video camera attached to an inverted microscope. Stomatal density was not affected in *era1-2*.

Example 5. Whole-cell patch clamp experiments.

Whole-cell patch clamp experiments were performed, and data were analyzed as described previously (Pei *et al.*, *Plant Cell* 9, 409 (1997)); the solutions used in patch clamp experiments contained 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA, 3.35 mM $CaCl_2$, 5 mM Tris-GTP, 5 mM Mg-ATP and 10 mM Hepes-Tris, pH 7.1 in the pipette, and 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Mes-Tris, pH 5.6 in the bath. For transient K+ current recordings, previously described standard solutions were used (Pei *et al.*, *Proc. Natl. Acad. Sci. USA* 95, 6548 (1998)). Abscisic acid ([±]-cis, trans-ABA; Sigma) was freshly added to the bath and pipette solutions; osmolalities of solutions were adjusted to 485 mmol/kg for bath and 500 mmol/kg for pipette by addition of D-sorbitol. In ~ 30 % of guard cells, anion currents did not respond to ABA or HFPA; for unbiased data analysis, non-responding cells were included in all reported data averages (Omission of non-responding cells would not change conclusions); statistical analyses were performed using EXCEL® (Microsoft); data are the mean ± SEM.

Example 6. Generation of fusion constructs.

ERA1-β-glucuronidase (GUS) fusion constructs were generated by inserting a 2.5 Kb PCR amplified genomic fragment of the ERA1 promoter into a promoterless GUS T-DNA plasmid (pBI121); this construct was transformed into the *Agrobacterium* strain LB4404. Transgenic plants were generated by vacuum infiltrating plants with *Agrobacterium* (Bechtold *et al.*, *C. R. Acad. Sci.* (Paris) 316, 1194 (1993)). Kanamycin resistant plants were selected in the next generation and intact whole leaves were tested for GUS activity using the fluorescent GUS substrate Imagene Green (Molecular Probes, Oregon). Seedlings were incubated in GUS-buffer for 2-4 hr at room temperature and then directly viewed under a microscope (25x) using blue excitation light. Positive fluorescent signal is yellow on a red chlorophyll autofluorescent background.

Example 7. Drought Measurement.

Transpiration rate and soil moisture were measured as previously described (Vartanian *et al.*, *Plant Physiol.* 104, 761 (1994)). For drought experiments, seeds of both wild-type (Col) and *era1-2* were germinated in individual pots each containing the same amount of prewetted soil. Plants were grown under constant light

(200 μmol m-2 sec-1) and watered by irrigation until just before the plants bolted (~ 3 weeks). Wild-type and *era1-2* plants (n = 16 each) were selected which were at the same developmental stages and had similar numbers of leaves. At this point, pots were removed from water and allowed to dry over time. Evaporation from soil was reduced by covering the soil surface with tinfoil so that water loss occurring primarily through plant transpiration could be quantified. Watered control plants were also analyzed. Pots were weighed every day at the same time. Pots containing no plants were subjected to the same treatments to determine the background rate of water loss.

Example 8. Inhibition of seed germination.

ABA inhibition of seed germination was analyzed as described previously (Koornneef, et al., Physiol. Plant 61, 377 (1984); Finkelstein and Somerville, Plant Physiol. 94, 1172 (1990)). Germination of sees was defined as positive when a radical tip had fully penetrated the seed coat (n=50 per condition). Each experiment (n = 25) included conditions comparing the indicated lines at multiple ABA concentrations.

The above disclosure is provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.